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Survival and Immune Response of Coho Salmon Exposed to Copper

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Copper concentrations of 10.1 ug/liter and higher caused significant mortality among coho fry during 30 days of exposure. The exposure of copper bioassay survivors to a natural challenge against V. anguillarum in seawater caused significant mortality among those fish from concentrations of copper at 13.9 ug/liter and higher. The reduced number of dead fish positive for V. anguillarum from the challenge suggests that sublethal copper stress and difficulty with seawater adaption may have caused several deaths.

Significant mortality occurred among coho fingerlings exposed to 24.6 ug/liter copper and higher for 31 days. Most of the survivors of these concentrations were unable to adapt to seawater and died within the first three days of challenge. Significant mortality also occurred during adaptation of survivors from 18.2 ug/liter copper where the mean mortality resulting from 31 days exposure was only 2%. The antibody level against V. anguillarum, measured by agglutinin titer, was significantly reduced in fish exposed to this concentration of copper when compared to that developed in control animals.

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SURVIVAL AND IMMUNE RESPONSE OF COHO SALMON EXPOSED TO COPPER

by

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FOREWORD

Effective regulatory and enforcement actions by the Environmental Protection Agency would be virtually impossible without sound scientific data on pollutants and their impact on environmental stability and human health. Responsibility for building this data base has been assigned to EPA's Office of Research and Development and its 15 major field installations, one of which is the Corvallis Environmental Research Laboratory, (CERL).

The primary mission of the Corvallis Laboratory is research on the effects of environmental pollutants on terrestrial, freshwater, and marine ecosystems; the behavior, effects and control of pollutants in lake systems; and the development of predictive models on the movement of pollutants in the biosphere.

This report describes part of the research on copper pollution being conducted at WFTS. Other reports are being prepared which describe additional research with copper, cadmium, and zinc.

A. F. Bartsch
Director, CERL

ABSTRACT

Vaccination with Vibrio anguillarum by oral administration during copper exposure and intraperitoneal injection prior to copper exposure was employed to investigate the effects of copper upon survival and the immune response of juvenile coho salmon (Oncorhynchus kisutch). Following copper exposure the survivors were challenged under natural conditions to V. anguillarum, the causative agent of vibriosis in fish.

Copper concentrations of 18.1 ug/liter and higher caused significant mortality among coho fry during 30 days of exposure. The exposure of copper bioassay survivors to a natural challenge against V. anguillarum in seawater caused significant mortality among those fish from concentrations of copper at 13.9 ug/liter and higher. The reduced number of dead fish positive for V. anguillarum from the challenge suggests that sublethal copper stress and difficulty with seawater adaptation may have caused several deaths.

Significant mortality occurred among coho fingerlings exposed to 24.6 ug/liter copper and higher for 31 days. Most of the survivors of these concentrations were unable to adapt to seawater and died within the first three days of challenge. Significant mortality also occurred during adaptation of survivors from 18.2 ug/liter copper where the mean mortality resulting from 31 days exposure was only 2%. The antibody level against V. anguillarum, measured by agglutinin titer, was significantly reduced in fish exposed to this concentration of copper when compared to that developed in control animals.

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SECTION 1

INTRODUCTION

The establishment of water quality standards to protect aquatic life is based largely upon criteria developed under laboratory conditions. Acute and chronic bioassays have been used to establish copper toxicity. Chronic tests are used to select a "no effect level" which is defined as the highest concentration that has no adverse effect on survival, growth, and reproduction. During these studies, environmental conditions are usually ideal when compared to those found in nature. Stress from environmental conditions can be an important factor in allowing an outbreak of infectious disease to occur among fish populations. Wild fish populations contain many fish which are resistant to disease but are carriers of pathogens capable of infecting nonresistant fish. These pathogens are released continuously into the aquatic environment and may survive long enough to infect other fish.

For many years marine aquaculture operations have suffered tremendous losses among cultured fish species from *Vibrio anguillarum*, the etiological agent of vibriosis. Renewed interest in mariculture of salmonid fish has prompted considerable research toward the control of this bacterial disease. Injected vaccines provide good protection and may be practical for fish farming operations but are impractical for hatchery-type production. Oral vaccine incorporated into the diet has been shown to be effective and may be an economical means of protecting juvenile fish against *V. anguillarum*. Both types of vaccine were employed in this study.

The purpose of this work was to determine the mortality from copper exposure and the effect of copper exposure on the immune response in juvenile coho salmon (*Oncorhyncus kisutch*). Continuous-flow diluters were used to expose the fish to constant concentrations of copper for approximately one month. The copper levels were designed to cover the range of concentrations from a no-effect level to that level causing nearly 100% mortality. A natural challenge to *V. anguillarum* was to be used to determine if the immune response was affected by the copper exposure. Antibody titers were measured in the injected fish to determine the magnitude of the immune response in fish exposed to the different copper concentrations and subsequent challenge.

SECTION 2

CONCLUSIONS

1. A sub-lethal concentration (18.2 µg/liter) of copper significantly reduced the level of antibody produced in fingerling coho salmon vaccinated by intraperitoneal injection.

2. Fingerling coho salmon, vaccinated or unvaccinated, surviving concentrations of 32.9, 24.6, and 18.2 µg/liter copper suffered significant mortality adapting to seawater.

3. Coho salmon fry, orally vaccinated during exposure to copper concentrations of 26.6, 18.1, and 13.9 µg/liter suffered significant mortality when placed in seawater.

4. The low percentage of coho salmon fry positive for V. anguillarum suggests that sub-lethal copper stress and poor sea water adaptation may have caused a number of deaths during the seawater challenge.

5. Copper concentrations of 18.1 µg/liter and higher caused significant mortality among coho salmon fry during 30 days of exposure to copper.

6. Fingerling coho salmon suffered significant mortality when exposed 31 days to copper concentrations of 24.6 µg/liter and higher.

7. Increased alkalinity and hardness decreased the toxicity of copper to coho salmon fry.

SECTION 3

RECOMMENDATIONS

Data from this study indicate that sublethal levels of copper interfered with both the immune response and the mechanism for seawater adaptation. Therefore, it is recommended that toxicity bioassays using smolt size anadromous fish be designed to test such latent physiological effects.

SECTION 4

LITERATURE REVIEW

TOXICITY OF COPPER TO FISH

Copper, in the form of soluble salts, naturally occurs in lakes and streams only in trace amounts up to about 20 µg/liter. Pollution from industrial uses, mine drainage, corrosion of copper and brass tubing, and aquatic herbicides probably accounts for the majority of the sources. Fish and other aquatic life are very sensitive to copper and considerable research has been conducted in this area. Doudoroff and Katz (1953), and McKee and Wolf (1963) have summarized earlier research.

Long-term or chronic exposure of fish to copper can produce sublethal effects which do not affect survival or appearance of adult fish but may affect spawning activity (Mount 1968; Mount and Stephan, 1969). McKim et al., (1970) studied the chronic effect of copper on several blood characteristics and were able to show that during the first 21 days of exposure to various copper concentrations the fish appeared to be under considerable stress as indicated by changes in the blood parameters. Most of the changes were not detectable after about 11 months exposure indicating a possible accommodation or adaptation.

HEAVY METALS AND THE IMMUNE RESPONSE

Heavy metals have been shown to cause other physiological and morphological changes in fish which could be used to detect heavy metal poisoning. Jacklm et al., (1970) reported changes in activities of five liver enzymes after exposing fish and fish liver homogenates to salts of various metals. Using killifish (Fundulus heteroclitus) exposed to 96 hour TL_m (50% mortality after 96 hours exposure) concentrations of copper, they demonstrated a reduced activity with most of the enzymes tested. Winter flounder (Pseudopleuronectes americanus) exposed to high and medium concentrations of copper exhibited morphological changes in gill, kidney, and liver tissue (Baker, 1969). The necrosis of kidney hemopoietic tissues suggested that copper induces hemolytic anemia as an initial lesion.

Suppression of some immune mechanisms has been demonstrated with several heavy metals. Nontoxic levels of lead increased the susceptibility of mice to Salmonella typhimurium (Hemphill et al., 1971). Mice were injected with different amounts of lead nitrate over a 30 day period. The percent mortality was directly related to lead intake when challenged with an LD_{50} (dose lethal to 50% of the animals) of a culture of S. typhimurium. The specific mechanism involved in this study was not identified.

There is a great amount of research today directed toward finding anti-tumor drugs which do not suppress the immune response. Suppression of anti-body plaque-forming cells with cis-Platinum (II) Diamminodichloride was demonstrated in mice by Khan and Hill (1971). It was thought that the anti-tumor effect of the platinum was due to stimulation of immunity, however, the study did not substantiate that idea. There was in fact a suppression of normal antibody forming cells. In a similar study Jones et al., (1971) investigated the effects of cadmium on the synthesis of specific antibody. Rats receiving cadmium one week and two weeks prior to antigen showed opposite antibody responses. Rats receiving cadmium two weeks prior to antigen had an enhanced antibody response. However, those injected one week prior to antigen had the response delayed and suppressed. The exact mechanism affected by cadmium was not identified.

The effects of copper on the immune response of vertebrates has received some attention through nutritional studies in small animals. Excess copper in a ration fed to mice reduced the phagocytic activity of leukocytes, the lysozyme titer and the bactericidal action of blood serum (Kolomiitseva et al., 1969). In most nutritional studies there appears to be an optimum level of copper which allows the maximum response of the mechanism involved.

Investigators working with drugs used to treat trypanosome infections found that colloidal copper interfered with the action of the drugs (Jancsó and Jancsó, 1934; Ormerod, 1961). It was determined that the copper blocked phagocytosis by the reticuloendothelial system which potentiated the action of the drugs.

STRESS AND DISEASE

Fish in natural water are continuously exposed to pathogens which are capable of causing disease. However, disease only occurs when the proper relationship between the fish, the pathogen, and the environment is established (Snieszko, 1974). Natural waters are not stable and can vary both physically and chemically depending upon natural factors or the activities of man. Many instances of pollution-caused fish kills lack specific data about the effects of certain chemicals upon the fish and how these chemicals may or may not allow invasion of the fish by disease organisms. A convincing observation on the combined effect of pollution, high water temperature, and low flow on fish deaths attributed to Aeromonas hydrophila was described by Pippy and Hare (1969). Migrating Atlantic salmon (Salmo salar) and some resident fish were affected by the outbreak. Toxic concentrations of copper and zinc coupled with low water level and high water temperature preceded the outbreak in the Miramichi River in New Brunswick, Canada. Isolates of A. hydrophila capable of killing test fish were isolated from moribund fish.

These kinds of environmental changes place fish in a state of stress which can be an important factor in the outbreak of infectious disease (Wedemeyer, 1970). When the stress requires an adjustment beyond the fish's capabilities, it is lethal, and while physiological disorders or disease result from sublethal stress, fish are not without some capacity to combat disease organisms. It is well established that they are capable of an immune response which can afford protection against certain pathogens (Ridgway et

al., 1966; Snieszko, 1970). A recent review by Corbel (1975) summarizes the current available information on the immune response in fish.

VIBRIOSIS

One particular disease commonly found in fish from marine environments is vibriosis. Vibrio-associated diseases have been described for many years in wild and captive populations of marine fish (summarized by Anderson and Conroy, 1970). The first report of vibriosis in Pacific salmon, specifically caused by V. anguillarum, was described by Cisar and Fryer (1969). An epizootic, which killed more than half a million juvenile fall chinook salmon, occurred in the Tint Slough salt water rearing impoundment near Waldport, Oregon. Pure cultures of V. anguillarum, capable of causing infection and death in inoculated test fish, were isolated from 70% of the dead fish examined. Research in the use of oral and injected vaccines for control of vibrio disease has shown encouraging results (Hayashi et al., 1964; Nelson, 1972; Fletcher and White, 1973; Rohovec et al., 1975). These investigators showed that both oral vaccination and vaccination by injection can provide good protection against vibriosis. High serum antibody titers were obtained by injection, resulting in fish more resistant to vibrio attack. Fryer et al., (1972) reviewed the etiology, pathology, and distribution of vibriosis. Control of the disease with drugs and results of early studies with oral vaccines are described in detail. Further research into the methods of vibriosis control, especially through oral immunization, were reported by Rohovec (1974). The investigator developed several bacterins which could be used both orally and parenterally. He determined that both wet-packed whole cells and formalin-killed lyophilized whole cells of the organism were effective immunogens. Intraperitoneal injection of formalin-killed whole cells mixed with Freund complete adjuvant provided protection against a natural challenge to V. anguillarum. Other parameters such as length of vaccination period, concentration of vaccine, and optimum water temperatures are described.

SECTION 5

METHODS AND MATERIALS

PRELIMINARY BIOASSAYS

Prior to beginning each immunization experiment, one or more preliminary bioassays were conducted to determine the range of copper concentrations to be used. Ten fish were used in the two highest concentrations in each bioassay. Each test was continued for at least 72 hours.

The apparatus used to expose coho fry to copper for both the preliminary bioassays and the oral immunization test was a proportional diluter similar to that described by Mount and Brungs (1967). Five concentrations and a background control were randomly assigned to duplicate sets of six aquaria. The aquaria measured 27 x 21 x 52 cm and each held approximately 27 liters of water. The diluter was designed to deliver one liter to each aquarium during every cycle (97 seconds/cycle) with a continuous flow to the aquaria. The copper stock solution was fed to the diluter from a marriotte bottle.

The apparatus used for copper exposure during the injected bacterin test as well as its preliminary bioassay was a continuous-flow serial diluter described by Garton (1976). The diluter was designed to deliver two liters per minute to each of 12 aquaria with continuous flow. Each aquarium measured 25 x 39 x 90 cm and contained approximately 74 liters of water. The stock solution of copper was introduced to the dilution apparatus by a metering pump. The stock solutions for all tests were prepared with copper chloride ($CuCl_2 \cdot 2H_2O$ reagent grade) and acidified with H_2SO_4 (0.1 ml/liter) to prevent precipitation. The stock solutions were prepared using well water.

Each aquarium was covered with 1/4-inch mesh plastic screen and the area around the sides of the aquaria was shrouded with black plastic curtains to prevent disturbance from room activities. Gentle aeration was provided to each aquarium to insure adequate oxygen levels. The photoperiod was controlled to simulate natural photoperiod during the spring months when use of the laboratory would cause unnatural lighting.

Water samples (500 ml) for copper analysis were collected twice a week from each concentration. Each sample was acidified with 12.5 ml of HNO_3 and evaporated to dryness. The samples were brought up to a volume of 25 ml with a 2.5% HNO_3 solution to give a 20 fold concentration. These samples were analyzed by flame atomic absorption spectrophotometry. Water samples for copper analysis taken during the injection bioassay were analyzed by flameless atomic absorption spectrophotometry. The water used for all bioassays

was obtained from wells 30 meters from the Willamette River at Western Fish Toxicology Station (WFTS), Corvallis, Oregon.

Dissolved oxygen, total hardness, alkalinity, and pH determinations were conducted daily. Measurements were conducted according to the American Public Health Association (1971). Temperatures were controlled and continually recorded during the copper bioassays.

COPPER BIOASSAY AND ORAL VACCINATION

Coho salmon fry, 45 to 50 mm in fork length with a mean weight of one gram, were obtained from the Fall Creek Hatchery, Oregon Department of Fish and Wildlife. They were trucked to the Western Fish Toxicology Station (WFTS) and held in a 200-gallon circular tank. The fish were fed Oregon Moist Pellet (OMP) diet for three days and then switched to Oregon Test Diet (OTD). The formulation of the OTD used was a modification [1] of that given by Lee et al., (1967). The diet was prepared in one-liter batches. Following overnight refrigeration, the gelled diet was sliced and forced through screens with 1/16-inch mesh. The cut pieces were weighed out in daily rations for each aquarium and frozen in plastic bags.

The OTD used to vaccinate the coho fry was prepared as above except for the addition of bacterin during the preparation. Formalin killed lyophilized whole cells of *V. anguillarum* from the Department of Microbiology at Oregon State University were used in preparation of the bacterin. The cells had been grown in Tryptic Soy Broth, formalin killed, washed in buffered saline, lyophilized, and stored under vacuum until used in the bacterin. [2]

One hundred fish were placed in each of 12 aquaria by stratified random assignment of groups of five fish. They were acclimated to the apparatus and fed OTD (at 6% of body weight) for six days. Beginning on the seventh day, the OTD with bacterin was fed and the copper exposure was initiated.

The copper solution was introduced into the diluter and the aquaria were allowed to come up to concentration. The 95% replacement time in the aquaria was approximately two hours. The diluter was designed to provide duplicate copper concentrations (40, 28, 19.6, 13.7, 9.6 µg/liter) and a background control. The aquaria were checked for dead fish before and after feeding each day. The OTD with bacterin was fed once a day slowly enough to allow total consumption of the diet. The amount of diet fed each aquarium per day was adjusted weekly according to the mortality incurred. This would provide equivalent amounts of bacterin per aquaria according to the number of fish present at various concentrations. Copper exposure was terminated following a 30-day exposure. The fish were then held under control conditions for a 10-day rest period and fed OMP. During this period the aquarium which received the highest concentration of copper, and where all fish had died, was rinsed thoroughly and supplied with freshwater without copper. One hundred stock fish were placed in this aquarium to serve as an unvaccinated control during the seawater challenge.

[1] Modification by J. H. Wales, Food Science and Technology, O.S.U.

[2] Bacterin provided by John Rohovec, O.S.U. Department of Microbiology.

After the 10-day rest period the fish were trucked to Tint Slough for a challenge to V. anguillarum. The Tint Slough facility at Waldport, Oregon is a marine rearing impoundment operated by the Oregon Department of Fish and Wildlife in which epizootics of vibriosis in juvenile chinook salmon (Oncorhynchus tshawytscha) have been reported, with mortality as high as 90% of the fish (Csaar and Fryer, 1969). The coho were placed in fiberglass tanks containing 60 liters of a 1:2 mixture of seawater and freshwater. After 20 minutes the mixture was brought up to 1:1. Following 30 minutes at 1:1, seawater was pumped directly into the tanks from the slough at a rate of four liters per minute. The challenge was continued for 25 days. The fish were fed OMP (all they would eat once a day), and dead fish were noted and removed twice a day.

Dead fish were placed in plastic bags, labeled, and stored on ice when examined the same day as removal. When examination was delayed, the fish were frozen until the day of necropsy. Examination of the dead fish included visual observation for gross pathological symptoms, aseptic dissection and bacteriological culture of kidney and liver tissue. Furunculosis and Brain Heart Infusion agar plates were innoculated and examined for typical colonies of V. anguillarum after 24 hours incubation at 30 C. Presumptive tests on typical colonies included microscopic examination of the gram reaction, and observation for morphology and motility using phase contrast microscopy. Suspected isolates were confirmed with V. anguillarum antiserum in rapid slide agglutination tests using the suspected isolates as antigen (Kolmer et al., 1951). Death was attributed to V. anguillarum when all these tests were positive.

COPPER BIOASSAY AND VACCINATION BY INJECTION

Coho salmon (mean weight of seven grams) raised at WFIS were selected for this study. These fish were obtained as eggs from the Fall Creek Hatchery, Oregon Department of Fish and Wildlife. One hundred fish were assigned to each of 12 aquaria as in the previous test. They were fed OMP (at 2% of body weight per day) throughout the acclimation period and bioassay.

On the sixth day, the fish were not fed and the next day the fish in tanks 7 to 12 were removed, anesthetized with MS-222 (Tricane Methanesulfonate)[1], and injected intraperitoneally with V. anguillarum bacterin. The V. anguillarum isolates were obtained from stock cultures in the Department of Microbiology at Oregon State University. Isolate C.S. 71 was cultured from spring chinook at Tint Slough in 1971. Stock cultures of this isolate were maintained in Cytophaga Seawater Agar deeps at 4 C (Pacha and Ordal, 1967).

Routine antigen preparation for mass cell culture, immunization, and agglutination were produced as follows. Typical V. anguillarum colonies were selected from Furunculosis Agar flats, examined for motility, and checked by gram stain and rapid slide agglutination.

[1]Kent Chemicals Ltd.

Confirmed colonies were used to inoculate Tryptic Soy Broth in 15ml test tubes. Following 24 hours growth, 2 ml were inoculated into 32-ounce bottle slants prepared with 100 ml of Furunculosis Agar.

After 48 hours at room temperature the cells were harvested with 10 ml of sterile phosphate buffered saline (PBS) (Williams and Chase, 1968) using glass beads to free the colonies. Before dilution to 50 ml with PBS the culture was checked for purity. Treatment beyond this step varied depending upon intended use.

For agglutination the cells were washed three times in PBS, centrifuged at 2500 rpm for 15 minutes, and resuspended in PBS to an optical density (OD) of 0.85 at 525 nm (approximately 5 x 10^9 cells/ml) as determined with a Bausch and Lomb Spectronic 20. Cells for agglutination were prepared and used on the same day.

The cell suspension used for injection was killed with 0.3% formalin and stored for 24 hours at 4 C before sterility was checked by inoculating two tubes of TS broth and two FA plates. If sterile, the cells were washed three times with PBS, resuspended to an OD of 0.85 at 525 nm and mixed 1:1 with Freund complete adjuvant (FCA). [4/] The cell suspensions and FCA were blended for seven minutes using a microhomogenizer. The blended vaccine was loaded into a 10-ml syringe which was used to fill 70 one-ml tuberculin syringes. Fish were injected just dorsal of the pelvin fin by the intraperitoneal (IP) route with 0.1 ml of the bacterin-FCA mixture. Anesthesia in an aqueous solution of MS-222 was necessary to facilitate handling without injury. After the injections were completed, the last fish injected was allowed to recover for 30 minutes before the copper solution was introduced. Nominal (expected) concentrations of copper were 35, 26.3, 19.7, 14.8, 11.1 ug/liter and a control. The duplicate tanks (1 to 6) were used as an unvaccinated control bioassay. These fish were not anesthetized or injected and were intended to demonstrate the effects of copper alone.

Copper exposure was continued for 31 days followed by a two-day rest period before transportation of the survivors to the Lint Slough facility for challenge. During the copper exposure at WFTS and challenge at Lint Slough the fish were fed OMP and the dead fish removed daily after feeding.

Transportation to Lint Slough was accomplished in 5 gallon carboys and required approximately 1½ hours time. Oxygen was bubbled into the containers during transport. Upon arrival at Lint Slough the fish were placed in the tanks and the seawater allowed to replace the freshwater as in the previous experiment. Salinity levels during this challenge period ranged from 24.1 to 26.4 parts per thousand with a mean of 25.3.

Vibrio-caused deaths normally occur in Lint Slough during the summer months when the temperature is above 12 C. An effective challenge

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[4/] Difco Laboratories

would normally produce heavy mortality between the fourth and tenth days after the fish are introduced to seawater. The water temperature at the beginning of this challenge was slightly above 12 C and dropped continuously until it reached 10 C by the tenth day of the challenge. Consequently the challenge was not very effective and the fish were sacrificed to determine if antibody titers had developed against V. anguillarum. Blood samples were drawn from sacrificed fish on the 16th and 34th day of exposure to seawater. On the second sampling day the remaining vaccinated fish and the unvaccinated fish held at 18.2 µg/liter copper were returned to freshwater. These fish were transported to the Fish Disease Laboratory of the Department of Microbiology and held in tanks similar to those used at Flint Slough. The remaining unvaccinated fish were counted and destroyed.

DETERMINATION OF AGGLUTINATION TITERS

The fish to be sacrificed were anesthetized and blood was collected by severing the caudal peduncle just posterior to the adipose fin. Blood was collected in 4 mm OD glass tubes, plugged with clay, and stored at room temperature for one hour prior to overnight refrigeration at 4 C. Following overnight storage the tubes were centrifuged at 1800 rpm for 10 minutes. After centrifugation the tubes were etched and broken at the serum clot interface and the serum drawn into 50 µl capillary tubes. These tubes were plugged with clay at the uncalibrated end and frozen for future use. During storage the capillary tubes were kept in test tubes sealed with Parafilm [b] to prevent sublimation.

Agglutinating antibody titers were determined by the microtiter method (Wittin, 1967; Benenson et al., 1968). Several styles of microtiter plates were tested for clarity of end points. A disposable V-bottom plate was selected since it had a highly transparent bottom. The determination of agglutination titers by the microtiter method provides a high degree of accuracy in titer determination from small serum samples. The precision of the microtiter method used is shown in Table 1.

TABLE 1. PRECISION OF MICROTITER AGGLUTINATION TEST USING A POOLED SERUM SAMPLE

Serum	Replications	Mean ($titer^{-1}$)	Standard error (Log_{10} value)	95% confidence interval ($titer^{-1}$)
Pooled sample	16	4277	.01081	3900-4690

[b] American Can Company

On the day of antibody titration, the capillary tubes containing serum samples were removed from the freezer and spread out in a horizontal position to prevent leakage during thawing. While these were thawing, 50 µl of 0.85 saline diluent were added to all the wells in one or two plates. When the serum had thawed, the plugged end of the 50 µl capillary tube was broken off and the tube drained to the index line. Often it was necessary to carefully expel bubbles which formed inside the tube during thawing.

The 50 µl serum sample was added to well number one and mixed by drawing the mixture into the capillary tubes several times. A small rubber bulb was used to facilitate accurate dispensing of the tube contents. The 50 µl microtiter diluters were heated to near incandescence, air cooled, wet in saline, and blotted before each dilution. Initially, each day, a blotter with calibrated circles would be used to check for proper delivery by each diluter. Following blotting, the diluter was gently placed in the first row of wells and allowed to take up the mixture. After gently mixing by four reciprocal rotations the diluters were placed in the second well and mixed as before. This procedure, repeated until the end of the plate, provided the two-fold dilutions. Following the titration the diluters were blotted, rinsed twice in saline and once in distilled water, then heated to near incandescence.

The *V. anguillarum* antigen, adjusted to 0.85 OD at 525 nm, was mixed and added to each well using a 50 µl pipetter. Three additional wells received only saline and antigen in order to serve as an auto-agglutination control. The plate was gently shaken in all directions and covered with an adhesive mylar sheet. These plates were incubated for two hours at room temperature and then stored for sixteen hours at 4 C prior to reading.

A uniform spread of agglutinated bacterial cells over the entire bottom of the well constituted a positive agglutination reaction. A button of cells at the very bottom of the V-shaped well constituted a negative reaction. The microtiter plates were read over a dark surface and checked with a broad field microscope for accuracy. During later titrations it was necessary to extend the dilution into a second plate in order to reach the higher end points.

STATISTICAL ANALYSIS

The copper concentrations and fish were assigned to aquaria by stratified random methods. Antibody titers determined by agglutination are reported as the reciprocal of the maximum dilution showing a positive reaction. Reciprocal titer values were converted to the $\log_2$ for statistical analysis. Means calculated on titer determinations after conversion to $\log_2$ were geometric and unpaired statistics were used for comparison of means. Statistical analysis of most of the data was completed using the CDC 3300 computer at Oregon State University.

SECTION 6

RESULTS

PRELIMINARY BIOASSAYS FOR THE ORAL VACCINATION EXPERIMENT

During the spring and fall, chemical parameters of the water supply at the WFTS vary considerably, depending on the amount of rainfall and the Willamette River stage. The alkalinity during the two preliminary bioassays ranged from 20 to 53 mg/l as $CaCO_3$ while the hardness ranged from 20 to 83 mg/l as $CaCO_3$ (Figure 1). Increased alkalinity and hardness decrease the toxicity of copper to fish (Stiff, 1971).

The alkalinity and hardness were decreasing during the early part of the first preliminary bioassay when nominal copper concentrations of 40 and 20 µg/liter were tested. The mortality during this bioassay (Figure 1a) indicated that the copper concentrations were too high to obtain the desired survival. The day the second preliminary bioassay was started, the alkalinity and hardness began to increase. During the next 72 hours both the alkalinity and hardness continued to increase and consequently the mortality at the nominal 20 µg/liter concentration during the second bioassay (Figure 1b) was considerably less than that observed during the first bioassay.

COPPER BIOASSAY AND ORAL VACCINATION

In this experiment coho salmon fry were orally vaccinated during a 30-day exposure to various concentrations of copper. Deaths were recorded daily through 30 days of exposure and the 10-day rest period. There were no survivors at a copper concentration of 34.4 µg/liter and only 7% survived at 26.6 µg/liter (Figure 2). Mean mortality at 18.1 µg/liter reached 41.5% and greater at higher copper concentrations, all of which were significant ($P = 0.05$) when compared to the control (Table 2). At the two lowest copper concentrations of 13.9 and 11.6 µg/liter the copper bioassay mortality was not significantly different ($P = 0.05$) from the controls.

The oral vaccination experiment was begun when the hardness and alkalinity were relatively high (79.5 and 53 mg/liter, respectively). These began to decrease and after six days were about 50% lower than at the beginning of the copper exposure. Mortality at the two highest copper concentrations was delayed, when compared to the preliminary bioassay data (Figure 1), and was probably due to hardness and alkalinity dynamics. The hardness and alkalinity remained low for the duration of the experiment as indicated by the means and ranges in Table 3. The measured copper concentrations are shown in Table 4.

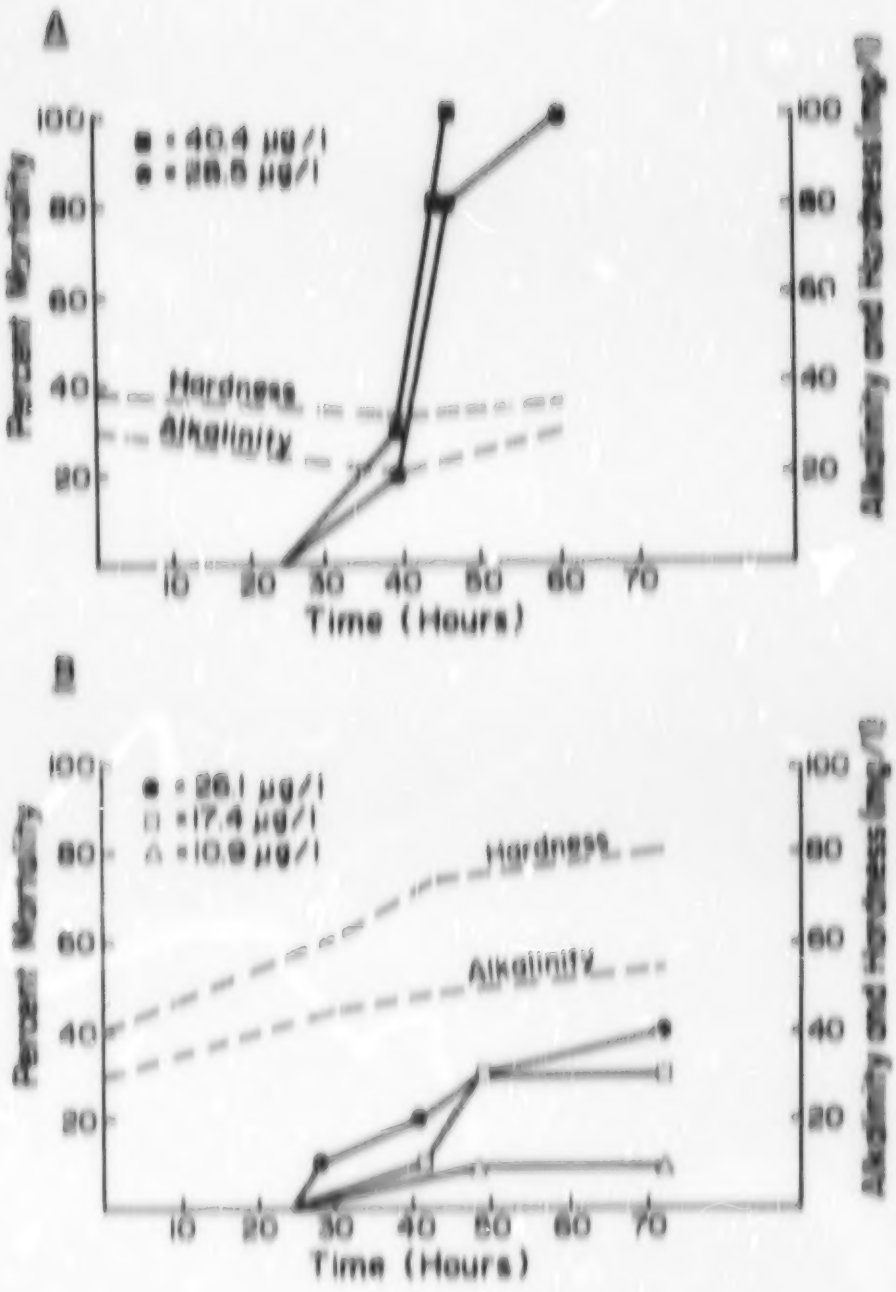

Figure 1. Mortality of coho fry exposed to copper during the preliminary bioassays conducted prior to the oral vaccination test with indicated variations in alkalinity and hardness.

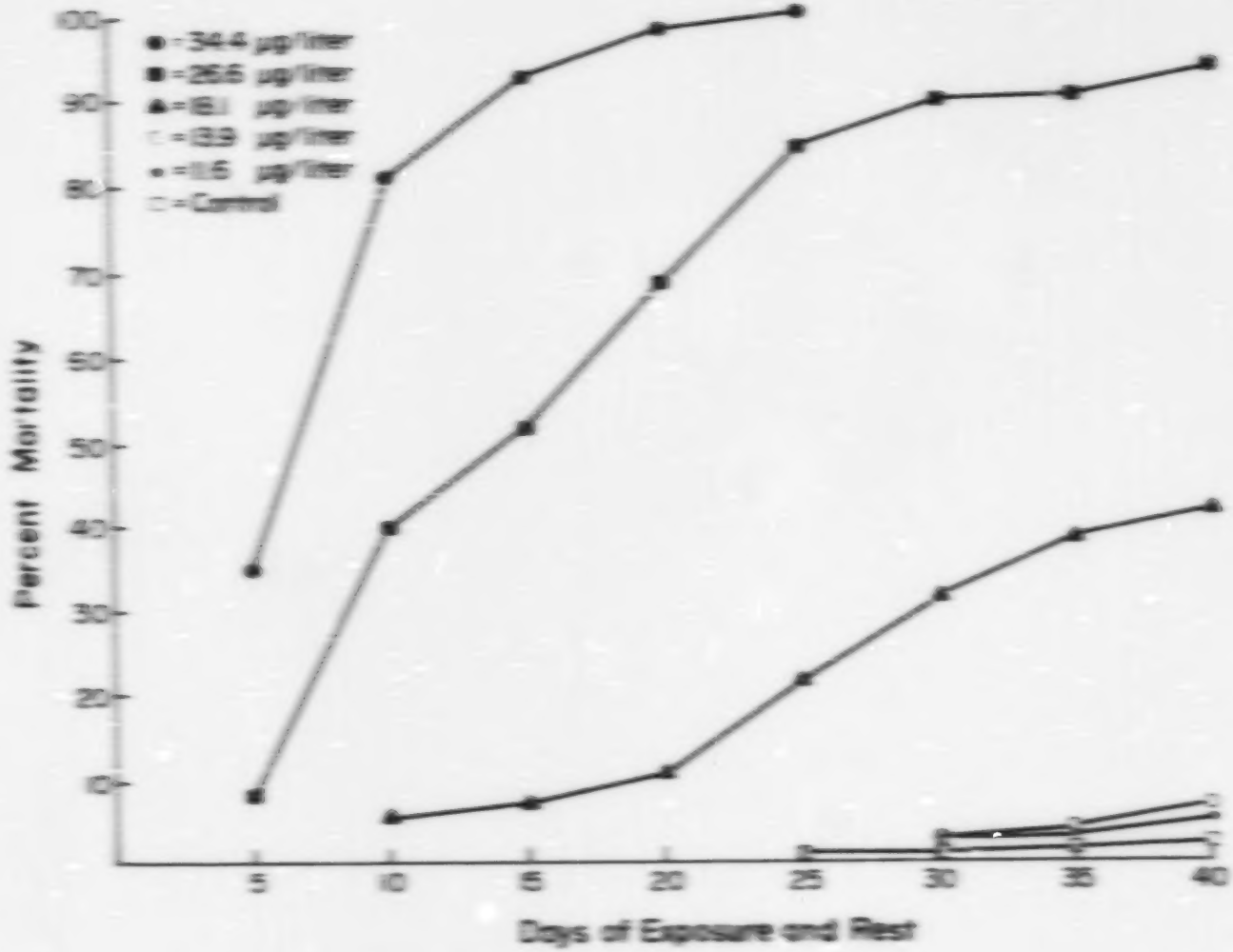

Figure 2. Mean mortality of coho salmon fry exposed to copper during the oral vaccination bioassay.

TABLE 2. EFFECTS OF ONE DAY EXPOSURE TO MIXTURES OF TWO ORGANIC SOLVENTS ON BODY WEIGHT AND RELATIVE LIVER WEIGHT

Mean Chamber Concentration (ppm)		Mean Terminal Body Weight (g)		Mean Relative Liver Weight (%)	
Toluene	Xylene	Male	Female	Male	Female
0	0	342	208	3.4	3.2
100	100	338	206	3.5	3.3
200	200	332	203	3.5	3.3
400	400	326	201	3.5	3.4
800	800	316	196	3.7	3.5

* Significantly different from the control value at $P \leq 0.05$

Animals were exposed for one day and were 10 weeks old at the start of exposure.

Characteristic	Number of Samples	Mean of Determinations	Standard Deviation	Range
Unconfined compressive strength (psi)	15	621	45.5	571 - 721
Splitting tensile strength (psi)	15	515	41.0	470 - 582
Flexural strength (psi)	22	582	41.4	519 - 638
Modulus of elasticity (ksi)	22	4.7	1.41	2.7 - 7.5
Poisson's ratio	22	.22	.07	.11 - .34

TABLE 1 CONCRETE CHARACTERISTICS OF THE CONCRETE USED IN THE CONCRETE BEAMS

TABLE 4. MEASURED TOTAL COPPER CONCENTRATIONS TAKEN DURING THE ORAL VACCINATION EXERCISE

Nominal Copper Concentrations (µg/liter)	Number of samples	Mean Measured Copper Concentration (µg/liter)	Standard Deviation	Range (µg/liter)
43	11	34.4	±3.1	27.2 - 38.1
28	12	25.6	±3.7	18.7 - 31.2
19.6	8	18.1	±2.8	13.5 - 19.7
13.7	6	13.9	±1.7	11.4 - 15.5
9.6	9	11.6	±1.7	8.6 - 13.8
Control	13	3.2	±1.4	1.3 - 6.0

One of the first indications of stress by copper was anorexia (loss of appetite). By the end of the first 24-hour period, fish in the 34.4 and 26.6 ug/liter tanks were eating an estimated 75% of their ration. After 48 hours, these fish were eating less than 50% of their food and fish at all levels of copper appeared to be in stress as indicated by the uneaten food in the aquaria. Control fish were eating 100% of the food being fed. The appetites of all groups of fish improved considerably during the rest period when they were fed OMP.

Following the 10-day rest period the fish were transferred to Lint Slough where fish began to die on the second day of exposure to seawater. Temperature and salinity varied daily, the magnitude of each being determined by the height of the tide and the amount of fresh water entering the slough (Table 5). The number of deaths continued to increase in all groups of fish exposed to copper during the seawater exposure.

The highest percent mortality among the fish challenged occurred in the unvaccinated control group (Figure 3). All groups of fish receiving bacterin were protected to some degree when compared to the unvaccinated control. Fish exposed to 13.9 ug/liter copper and above suffered significant mortality ($P = 0.05$) when compared to the control, while those exposed to 11.6 ug/liter had similar mortality to the control, indicating that the lowest copper level had little, if any, effect on the immune response and subsequent survival. Fish surviving copper exposure at higher levels may have had difficulty adapting to seawater, however it is difficult to attribute death to this one factor. Many of those fish which died during challenge from concentrations of 13.9 ug/liter copper and above were very thin and apparently never went back to feeding and may have died from starvation. The percentage of thin fish ranged from 18 to 47% among the dead fish from 11.6, 13.9, and 18.1 ug/liter copper.

The results of the examination of dead fish indicate that, with the exception of the fish held at 26.6 ug/liter, 50 to 63% of the vaccinated fish that died were positive for V. anguillarum (Table 6). Many of the fish that were negative for V. anguillarum were the debilitated fish that never began to eat again after copper exposure. These fish, though very debilitated, could have been protected through the small amounts of vaccine they ingested during copper exposure. It is assumed that the remainder of the fish negative for V. anguillarum were not infected and died from the stress of copper exposure and seawater adaptation. The percentage of recovery of V. anguillarum from fish tissues was very high in the unvaccinated control group which received no vaccine and were not exposed to copper.

PRELIMINARY BIOASSAY FOR THE VACCINATION BY INJECTION EXPERIMENT

The preliminary bioassay for this experiment was conducted for seven days. Ten fingerling coho were placed in each aquaria and a copper stock solution prepared to deliver concentrations of 50, 35, 24.5, 17.1, and 12.0 ug/liter copper. Within 72 hours, 100% of the fish were dead at 50 ug/liter and at the end of the 7-day test, 40% of the fish were dead at 35 ug/liter (Figure 4). The latter concentration was chosen as the highest concentration in the injection experiment.

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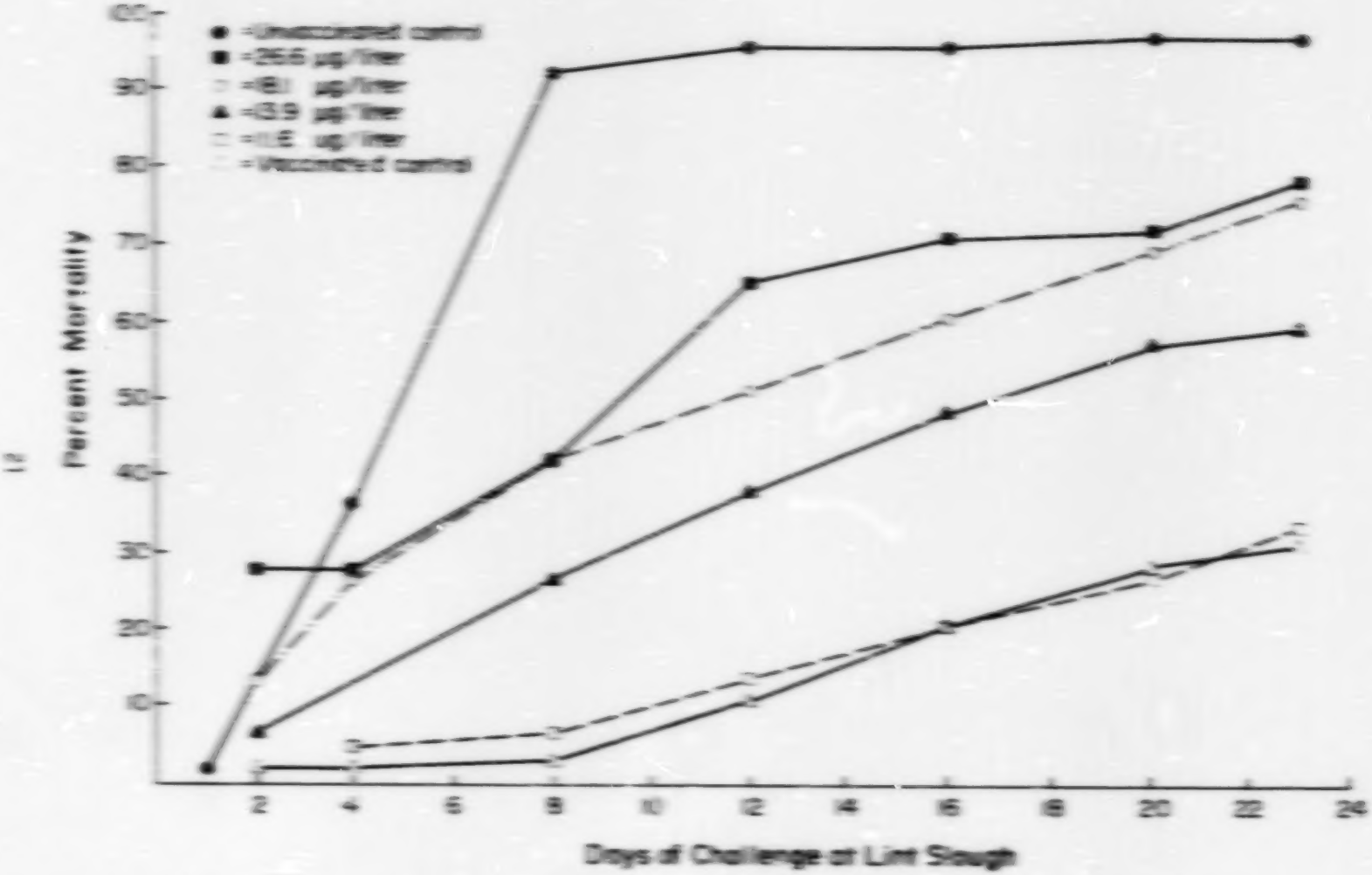

Figure 1. Mean mortality of coho salmon fry during natural challenge to *V. anguillarum* following 10 days exposure to copper.

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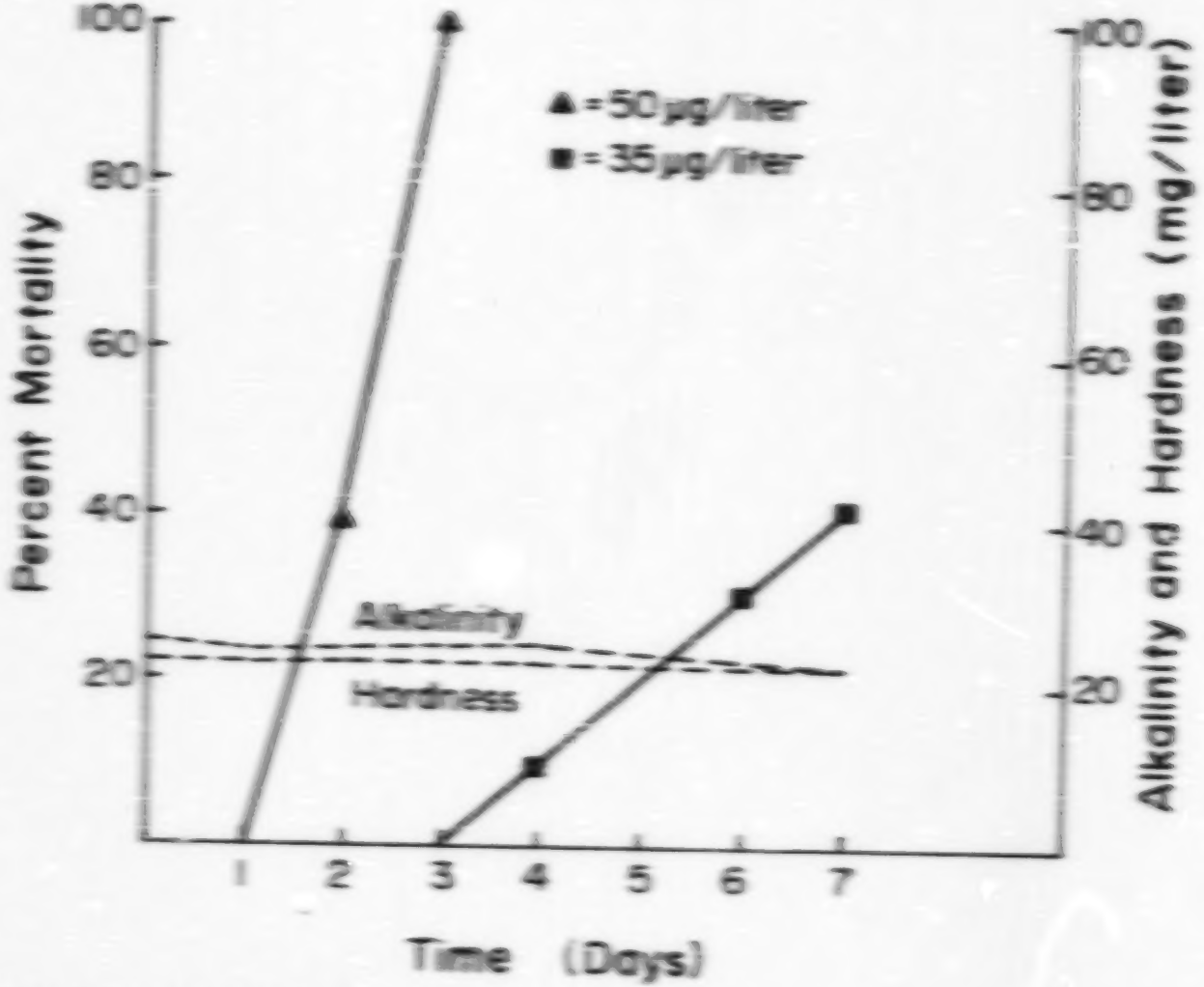

Figure 4. Mortality of coho fingerlings exposed to copper during the preliminary trial step for the vaccination by injection test with variations of alkalinity and hardness.

TABLE 7. MEASURED TOTAL COPPER CONCENTRATIONS FROM EXPERIMENTAL TANKS DURING THE INJECTED BACTERIA ASSESSMENT

Nominal Copper Concentration (µg/liter)	Number of Samples	Measured Copper Concentration (µg/liter) Mean ± S.D.	Range (µg/liter)
35	24	32.9 ± 4.0	27.8 – 38.8
26.3	24	24.6 ± 3.7	16.3 – 29.0
19.7	24	18.2 ± 2.2	12.2 – 21.6
14.8	24	13.6 ± 1.7	9.2 – 15.7
11.1	24	10.1 ± 1.4	6.9 – 11.6

TABLE 8. CHEMICAL CHARACTERISTICS OF THE WATER IN THE EXPERIMENTAL TANKS DURING THE INJECTED BACTERIA BIOASSAY

Characteristic	Units	Number of Analysis*	Determinations Mean ± S.D	Range
Dissolved Oxygen	mg/liter	223	9.1 ± 0.5	7.4 – 10.6
pH		36	7.2** ± 0.1	6.7 – 7.4
Alkalinity	mg/liter as $CaCO_3$	30	21.1 ± 2.0	13.0 – 26.0
Hardness	mg/liter as $CaCO_3$	36	21.7 ± 1.1	20.0 – 25.0
Temperature	C	38	12.2 ± 0.3	11.5 – 12.8

* Analysis conducted at least weekly in each concentration

** Native

COPPER BIOASSAY AND VACCINATION BY INJECTION

In this bioassay the coho fingerlings, after being injected with the V. anguillarum bacterin, were exposed for 31 days to copper concentrations ranging from 32.9 to 10.1 ug/liter (Table 7). This experiment was conducted in early fall resulting in relatively stable alkalinity and hardness values (Table 8). Significant mortality occurred only at the two highest copper concentrations and no significant differences in mortality were observed between vaccinated and unvaccinated fish held at any copper concentration (Table 9). A significant proportion of the mortality occurred between the fifth and fifteenth day of the bioassay.

During the copper bioassay the appetites of the fish at the higher concentrations diminished, but the fish remained in good condition, showing no signs of disease at the end of the exposure period. Fish were held for 48 hours following termination of the copper exposure before being transported to Lint Slough for the seawater challenge. By the end of the 48 hour rest period all fish appeared to be feeding normally and only one fish died during that period.

During the first two days of seawater exposure heavy mortality occurred among the survivors of the copper bioassay, especially in the groups which were exposed to the highest copper concentrations (32.9 and 24.6 ug/liter) (Table 9). All of the fish that died during the first three days were negative for V. anguillarum as were most of the fish that died during the first seven days of seawater exposure. The lack of Vibrio ascribed deaths in the unvaccinated fish during the period from the fourth to seventh day indicated the challenge was insufficient. The greatest number of dead fish positive for V. anguillarum occurred among the fish exposed to 18.2 ug/liter copper.

Temperatures by the eleventh day of the challenge had dropped from 12.2 to 10.0 C and only occasional deaths were appearing throughout all groups. The number of deaths, regardless of cause, reflected the concentration of copper in both the vaccinated and unvaccinated groups.

On the 16th day of the challenge, between 25 and 30 fish were sacrificed from the vaccinated controls and the group of fish exposed to 18.2 ug/liter copper. The blood was withdrawn and prepared for antibody titration. Analysis indicated that antibody titers had developed sufficiently so that they could be measured and therefore it was decided that all the fish would be removed from the seawater for antibody analysis. Similar samples to those taken above were removed from the groups exposed to 13.2 and 10.1 ug/liter copper just prior to transporting the fish back to the laboratory on the 34th day of exposure to seawater.

ANTIBODY TITERS DEVELOPED FOLLOWING INJECTION, COPPER EXPOSURE, AND SEAWATER CHALLENGE

Blood samples for antibody titer determination were collected at two different times. The first samplings, described above, were taken during the seventh and ninth weeks after injection. The remainder of the blood samples were collected 16½ weeks after injection. A significant increase in antibody

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titers occurred between the first and last sampling times (Table 10). At both sampling times the antibody titers at 18.2 µg/liter copper were significantly lower than the controls. At the last sampling the mean titer for those fish from 10.1 µg/liter copper was considerably lower than the controls, while those at 13.6 µg/liter copper were equal to the controls, indicating that the copper may have enhanced antibody production.

A sample of 20 unvaccinated fish was taken during the second sampling from the group of fish held at 18.2 µg/liter copper to be used to establish the background titers present in the fish. The mean reciprocal titer from these fish was 19.9 dilutions with a range of 8 to 32 dilutions. Less than 4% of the titers from vaccinated fish fell within this range and were considered to be a result of poor immune response or more likely an improper injection. The day after injection several aquaria had small amounts of adjuvent floating on the surface indicating that some injections were made into the intestinal tract instead of into the peritoneal cavity.

SECTION 7

DISCUSSION

Because the water supply at the Western Fish Toxicology Station does not have a constant level of alkalinity and hardness during the rainy season and these factors affect copper toxicity, preliminary bioassays were necessary to establish a satisfactory level of copper for each test. Mortality observed in the preliminary bioassays with coho fry indicated what effect alkalinity and hardness would have on the toxicity of copper to fish. Lloyd and Herbert (1962) found a linear relationship between hardness and the lethal threshold concentration for rainbow trout. Stiff (1971) suggested that the difference in the toxicity of copper in hard and soft water may be due to a copper carbonate complex which is less toxic than the free cupric ion and whose formation is favored at higher alkalinities. The decreasing alkalinity and hardness during the first five days of the oral immunization bioassay increased the toxic effect of the copper to the fry and resulted in higher mortality at both 34.4 and 26.6 µg/liter copper.

The first test conducted was designed to determine what effect copper exposure would have upon the protection provided to coho salmon fry by oral immunization with a V. anguillarum bacterin. The fish were fed bacterin daily while being exposed to various copper concentrations. McKim and Benoit (1971) found that 13.5 µg/liter copper had a severe effect on survival of juvenile trout where slightly less than three months exposure resulted in 15% mortality in water with an alkalinity and hardness between 40 and 45 mg/liter. The data from the coho fry bioassay indicate that 18.1 µg/liter has a definite effect (mean=42% mortality) on survival of coho fry exposed to copper for 30 days. Mortality continued during the ten day rest period, but appeared to be reaching an asymptote by that time.

Looking at the effects of copper upon challenge survival, the level of significant effect begins at 13.9 µg/liter. The stress of copper to these fish, and the additional stress of seawater and possibly vibriosis, produced further mortality. They could also have died from starvation whether or not they were protected. The development of salinity tolerance in coho salmon has been shown to occur in the fry stage and is related to fish size (Conte et al., 1966). Coho fry 3 to 4 cm in length had a median survival time greater than 30 days when held at a salinity of 26 parts per thousand. Therefore, these fish under normal conditions should have been able to survive the transfer to seawater.

In the study using coho fingerlings vaccinated by injection, mortality at the two highest copper concentrations (32.9 and 24.6 µg/liter) was

significantly greater than the controls ($P = 0.05$). The few deaths occurring at 18.2 µg/liter copper and below suggest that these concentrations are safe (non-lethal) for fingerling coho salmon under similar water conditions.

When the survivors from the two highest concentrations were placed in seawater nearly all the fish died within three days. This time period is generally not long enough for *V. anguillarum* infection to cause death. This was supported by the fact that all the dead fish were negative when examined for *V. anguillarum*. The survivors at 18.2 µg/liter copper also suffered significant losses but fish at the lower copper concentrations and control fish suffered little mortality from stress of placement into seawater. The data indicate that concentrations of copper down to 13.6 µg/liter which appear to be non-lethal levels for coho fingerlings can inhibit their ability to adapt to seawater. Recent studies conducted by Lorz and McPherson (1976) produced similar results. Yearling coho, exposed to 20 µg/liter copper for 144 hours without any mortality, suffered nearly 40% mortality when placed in seawater. Longer exposure to copper at 20 µg/liter caused only slight mortality in freshwater but increased the mortality significantly in seawater.

The efficacy of the challenge diminished considerably as the water temperature dropped to near 10 C. The number of deaths following acclimation failed to increase and there was little difference in the number of deaths between vaccinated and unvaccinated fish. Since the fish were injected with a bacterin, a humoral antibody response had been initiated and could be measured by titration. The remaining fish were sacrificed and the blood collected for antibody measurement.

Antibody titrations conducted on unvaccinated fish indicated the presence of natural antibody titers which were at 32 dilutions or below. There were also a few vaccinated fish with titers at that level. These, in part, would result when the inoculum was injected into the intestine by mistake, initiating little, if any, measurable immune response. There is also a certain portion of any population that is immunologically incompetent. It is also quite possible that in some fish the stress involved in handling as well as the copper exposure completely blocked the immune response.

The means of the agglutinin titers of serum samples taken 50 days after vaccination from fish exposed to 18.2 µg/liter copper indicated a significant ($P = 0.05$) reduction in antibody concentration. This reduction prevailed throughout the entire vaccination period (115 days) in the group of fish held at 18.2 µg/liter of copper. However, at 13.6 µg/liter copper exposure, there is an enhanced antibody response compared to that of the fish held at 10.1 µg/liter copper.

The exact mechanism involved in the reduction of the antibody titer is not known. Further research would be needed to determine if copper directly inhibits antibody production or whether chronic stress caused by the copper is directly involved with the reduced antibody production. The metabolic changes which take place during stress are directed toward increasing the possibility of survival; however, these changes often make the organism more susceptible to infection (Wedemeyer, 1970). Studies conducted on fish in stress indicate that the metabolic changes which take place are similar to

those of higher vertebrates (Hoar, 1957). In studies conducted with sockeye salmon, Fagerlund (1967) demonstrated that stress from fright and handling increased the level of corticosteroids present in the fish. Little work, however, has been done with fish to relate levels of corticosteroids with various aspects of the immune response such as antibody formation.

As pollution control methods improve there will undoubtedly be fewer catastrophic fish kills by heavy metals such as copper. However, as these studies indicated, sublethal levels of copper or other pollutants may have a significant effect upon physiological responses such as acclimation to seawater and may interfere with a successful immune response to disease organisms.

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